ELSEVIER

International Journal of Pharmaceutics 108 (1994) 49–55

international journal of pharmaceutics

Solution behavior of leuprolide acetate, an LHRH agonist, as determined by circular dichroism spectroscopy

Michael E. Powers [a], Akwete Adjei [b], Mou-Ying Fu Lu [b], Mark C. Manning [a,*]

[a] *School of Pharmacy, Campus Box C238, University of Colorado Health Sciences Center, Denver, CO 80262, USA,*
[b] *Pharmaceutical Product Development, Department 493, Abbott Laboratories, North Chicago, IL 60064, USA*

(Received 4 October 1993; Modified version received 10 January 1994; Accepted 20 January 1994)

Abstract

Leuprolide acetate, a potent agonist of luteinizing hormone-releasing hormone (LHRH), was found to display little or no secondary structure in aqueous solution as determined by circular dichroism (CD) spectroscopy. However, upon addition of trifluoroethanol, CD spectra consistent with type II β-turn structures were observed. CD spectroscopy was also employed to evaluate the aggregation of leuprolide in solution. In aqueous solution, leuprolide does not readily aggregate. However, in ethanol/water mixtures, concentration- and temperature-dependent aggregation was observed. This demonstrates that CD spectroscopy can be an effective analytical tool for assessing not only the structure of peptides, but also detecting peptide aggregation.

Key words: Leuprolide; Aggregation; CD spectroscopy; Secondary structure; LHRH agonist; Nonaqueous solvent

1. Introduction

Leuprolide acetate (LPA) is a luteinizing hormone-releasing hormone (LHRH) agonist with a sequence similar to that of other LHRH analogs (see Table 1). Numerous reports have appeared regarding the stability of other LHRH-like molecules (Winterer et al., 1983; Shi et al., 1984; Johnson et al., 1986; Hahn et al., 1987; Helm and Müller, 1990; Motto et al., 1991; Okada et al., 1991; Oyler et al., 1991; Powell et al., 1991), focusing on the chemical degradation of LHRH compounds. However, little is known about the structure and physical stability of LHRH analogs in solution. This study describes the secondary structure and aggregation behavior of LPA, both in aqueous solution and in the presence of non-aqueous solvents.

2. Materials and methods

All CD spectra were measured on an Aviv 62DS CD spectrophotometer equipped with a thermoelectric temperature control unit. Temperatures are accurate to ± 0.2°C. Strain-free quartz cells were purchased from Hellma, and pathlengths ranged from 0.5 to 10 mm. Data collection employed a 0.25 nm step size, a bandwidth of

* Corresponding author.

SSDI 0378-5173(94)00020-6

1.0–1.5 nm, and an averaging time of 3 s per data point.

Particle size analyses employed a Nicomp 370 submicron particle sizer. Measurements were made at room temperature over a period of 10 min. Samples were placed in 0.5 ml cuvettes.

Trifluoroethanol (Aldrich) was used without further purification. Leuprolide acetate was provided by Takeda Abbott Pharmaceuticals. Peptide concentrations were determined using an extinction coefficient of 0.53 at 277 nm for a 1 mg/ml aqueous solution with a pathlength of 1 mm. The extinction coefficient in 20% ethanol was not found to be appreciably different (< 10% difference). Experiments were performed both with leuprolide acetate solutions where no buffer was added and with 10 mM phosphate buffer, and no significant difference was observed.

3. Results and discussion

3.1. Secondary structure of LPA in aqueous solution

Although CD spectra of LHRH, LHRH analogs, and fragments have been reported (Marche et al., 1976; Cann et al., 1979), little is known regarding the structure of LHRH agonists in solution. Therefore, we have initiated structural studies on LPA examining the far UV (λ = 175–250 nm) CD spectrum. In aqueous solution, LPA does not appear to adopt a significant amount of a preferred secondary structure (Fig. 1). However, it is difficult to determine exactly the types and amounts of any secondary structure due to large contributions from the Tyr and Trp side chains.

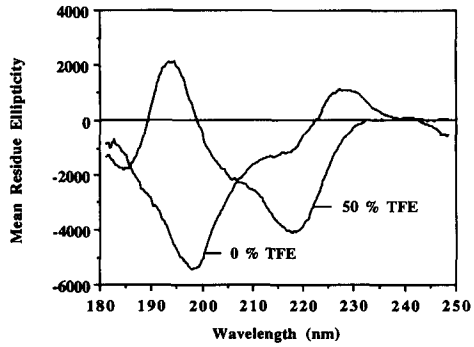

Fig. 1. Far UV CD spectra of LPA in water and in 50% TFE/water (v/v). The concentration of LPA was 0.1 mg/ml and the pathlength was 1 mm.

Many peptides which do not adopt a regular structure in aqueous solution do so upon the addition of organic solvents. One common technique for stabilizing latent secondary structure in peptides is to add perfluorinated alcohols, such as trifluoroethanol (TFE), a strong hydrogen bond donor. It has been shown that TFE can dramatically stabilize secondary structures in small peptides, particularly of α-helical conformations (Holladay, 1977; Wu and Yang, 1981; Manning, 1989; Lehrman et al., 1990; Honda et al., 1991; Matsuura and Manning, 1993). Increasing the concentration of TFE from 0 to 50% (v/v) leads to a marked change in the far UV CD spectrum (Fig. 1). The difference spectrum (50% minus 0%) displays three distinct features: a strong negative band at 220 nm, a strong positive band near 195 nm, and a weak negative band at 180 nm (Fig. 2). Such a CD pattern has been termed a class B spectrum by Woody (1974), and is indicative of formation of a β-turn. It has been shown that class B spectra are observed for peptides

Table 1
Sequences of LHRH and related analogs

Peptide	Sequence
LHRH	pGlu His Trp Ser Tyr Gly Leu Arg Pro Gly NH_2
LPA	pGlu His Trp Ser Tyr Gly Leu Arg Pro NHEt
Fertirelin	pGlu His Trp Ser Tyr Gly Leu Arg Pro NHEt
Histrelin	pGlu His Trp Ser Tyr Gly Leu Arg Pro NHEt
Nafarelin	pGlu His Trp Ser Tyr Gly Leu Arg Pro Gly NH_2
Triptorelin	pGlu His Trp Ser Tyr Gly Leu Arg Pro Gly NH_2

Leu, D-leucine; Trp, D-tryptophan; Nal, 2-naphthylalanine; pGlu, pyroglutamate.

which adopt type II turns (Perczel and Fasman, 1992; Perczel et al., 1992). Similar spectra have been observed for delta sleep-inducing peptide (DSIP), a nonapeptide which partially exists in aqueous solution as a folded conformation with two type II turns (Gray et al., 1994). These experiments suggest that nascent secondary structure exists in LPA, and that the structures can be stabilized by the addition of TFE.

3.2. Aggregation of LPA as determined by CD spectroscopy

The major physical instability exhibited by LHRH analogs is aggregation (Powell et al., 1991). For the purposes of this study, aggregation is taken to refer to association of the peptide on a microscopic level (Manning et al., 1989), meaning the aggregates remain in solution, but with chemical and physical properties differing from the dispersed monomeric species. Therefore, aggregation is a process distinct from precipitation.

Analysis of peptide aggregates is difficult, as the primary particles are typically too small for detection by light scattering methods. Therefore, we have chosen to monitor the near UV (NUV, $\lambda = 350–250$ nm) CD spectra of LPA as a function of concentration in order to detect aggregate formation. Aggregation should alter the band shape and intensity of near UV CD bands (Manning, 1992).

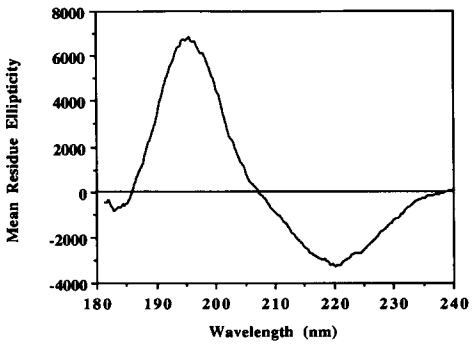

Fig. 2. Difference spectrum of LPA in water and in 50% TFE/water (v/v). The spectrum is plotted as the 50% TFE/water minus the spectrum in water (0% TFE).

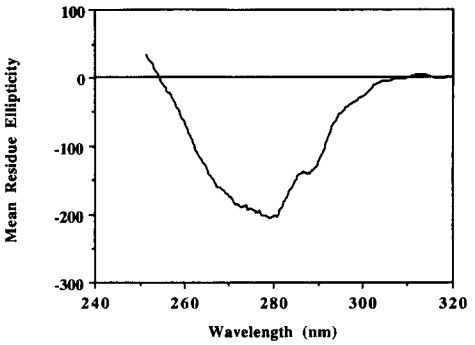

Fig. 3. Near UV CD spectrum of LPA in water (5 mg/ml). The pathlength was 1 mm and the sample temperature was 25°C.

The search for nonparenteral routes of administration of peptides and proteins has led to new formulation demands. Certain formulations designed for transdermal delivery of LPA include high concentrations of ethanol, a solvent in which LPA is readily soluble (Lu et al., 1992). However, the behavior of these formulations can be erratic. We hypothesized that the inconsistency of the formulations might be due to formation of small soluble aggregates, as the concentrations frequently exceeded 10 mg/ml (Lu et al., 1992).

Solutions of LPA in water and in 4:1 ethanol/water mixtures were prepared at known concentrations. In aqueous solution (pH 6), no evidence of aggregation can be detected by CD within the concentration range studied (up to 20 mg/ml). Both the intensity and band shape remained essentially constant for aqueous LPA solutions of concentrations up to 10 mg/ml. A representative CD spectrum is shown in Fig. 3. However, in 4:1 ethanol/water mixtures, aggregation of LPA could be detected.

Initial studies were conducted with CD sample temperatures at 5°C. Below concentrations of 3 mg/ml, the CD spectra appeared as a single negative maximum centered at 275 nm with an intensity of approx. -250 degree cm^2 dmol^{-1} (Fig. 4). Increasing the concentration to 4.2 mg/ml produces a sharp change in the CD spectrum. It then displays two negative bands, a peak at 270 nm and a shoulder at 280 nm. At a concentration of 5.45 mg/ml, these two features

have become distinct negative maxima at 268 and 288 nm. Variation of the CD spectrum with increased concentration is indicative of aggregate formation. Therefore, aggregation of LPA is accentuated by the presence of large amounts of nonaqueous solvents.

The presence of two bands suggests that, as LPA associates, the local environment around the aromatic groups differs widely. In general, two types of sites are generated, one where the side chain is relatively solvent accessible and one where it is not. In addition, the packing in the aggregate may also alter the preference in side chain dihedral angle, which will affect the NUV CD spectrum as well. Under these conditions, the far UV spectrum does not change, indicating that the effects do not arise from a transition in secondary structure.

Similar aggregation behavior was observed for samples held at 25°C. The overall intensity for monomeric LPA was lower (-150 degree cm^2 dmol^{-1}) than at 5°C, but the band shape and position (275 nm) were the same (Fig. 5). The effect of temperature on aggregation of LPA is pronounced. Upon increasing the temperature to 25°C from 5°C, the concentration required to aggregate LPA is nearly doubled (cf. Fig. 4 and 5). This suggests that there may be only a small activation energy to aggregation of LPA. This aspect of its stability will be the focus of further studies. Dynamic light scattering measurements indicated that the aggregates were less than 0.01 μm in diameter, the lowest detectable limit using the Nicomp particle size analyzer. Therefore, detection of these small aggregates can only be accomplished with methods such as CD spectroscopy.

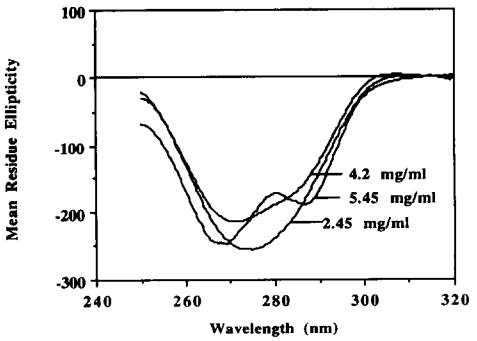

Fig. 4. Concentration dependence of the near UV CD spectrum of LPA in a 4:1 ethanol/water mixture. The pathlength was 1 mm and the sample temperature was 5°C.

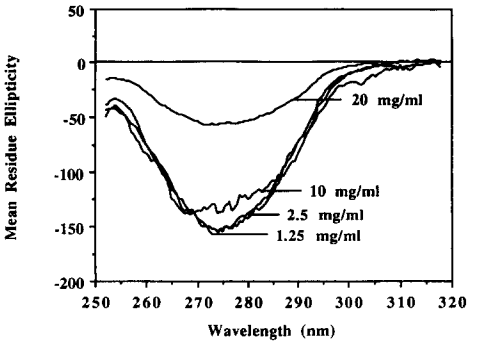

Fig. 5. Concentration dependence of the near UV CD spectrum of LPA in a 4:1 ethanol/water mixture. The pathlengths were either 0.5 or 1 mm and the sample temperature was 25°C.

Surface tension measurements are another tool which has been employed to detect peptide association (Powell et al., 1991). It has been reported that certain LHRH agonists are surface active, as determined by surface tension measurements (Powell et al., 1991). Detirelix, for example, is highly hydrophobic and has been found to have a critical micelle concentration of 2 mg/ml (Powell et al., 1991). The degree of activity varies greatly depending upon the sequence, as nafarelin did not form micelles even at concentrations above 10 mg/ml. Such information is important for proper handling of LPA during isolation and purification, as well as indicating its propensity to adsorb to surfaces and form aggregates in solution. Measurements of the surface tension of LPA do not indicate micelle formation, for concentrations up to 10 mg/ml, so any aggregation occurring must be at a concentration below the critical micelle concentration.

Aggregation in neat ethanol appears to be much more facile than in 4:1 ethanol/water (Fig. 6). Whereas it requires concentrations of nearly 10 mg/ml to aggregate at room temperature in 4:1 ethanol/water, it requires only ~ 1 mg/ml concentrations in ethanol. In addition, the sign of the near UV CD band of LPA changes upon

increasing the ethanol content of the solvent from 80 to 100%. These data suggest that aggregation in water would require very high concentrations.

3.3. Effect of excipients on the aggregation of LPA

A number of additives have been reported to increase the stability of proteins (Wang and Hanson, 1988). Additionally, a number of additives have been shown to enhance the percutaneous absorption of LPA (Lu et al., 1992). However, little is known regarding the effect of additives on aggregation. In order for CD to be a useful analytical tool, it should be able to analyze formulations as well as bulk drug material.

Polymers are a common class of excipients (Wang and Hanson, 1988) and polyvinylpyrrolidone (PVP) was chosen as a representative case. The effect of PVP on the concentration-induced aggregation of LPA in 4:1 EtOH/water mixtures was followed by CD spectroscopy as detailed above (see Fig. 7). The concentration of PVP was 1% (w/v). It appears that PVP may slightly promote LPA aggregation, as CD changes occur at slightly greater concentrations than without PVP added. It does demonstrate that the aggregation of LPA can be detected using NUV CD spectroscopy without interference from additives like PVP. Similar measurements were made for oligosaccharide containing solutions, again without interference (results not shown). Provided that the excipient did not exhibit significant absorption in the NUV, any formulation could be analyzed by this method.

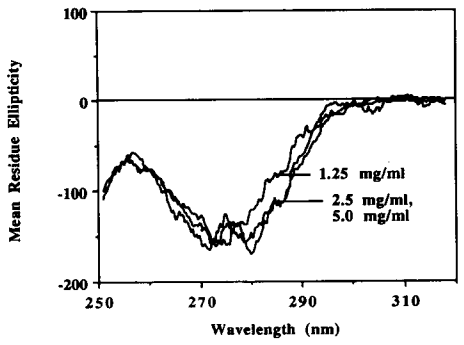

Fig. 6. Concentration dependence of the near UV CD spectrum of LPA in neat ethanol. The pathlengths were either 0.5 or 1 mm and the sample temperature was 25°C.

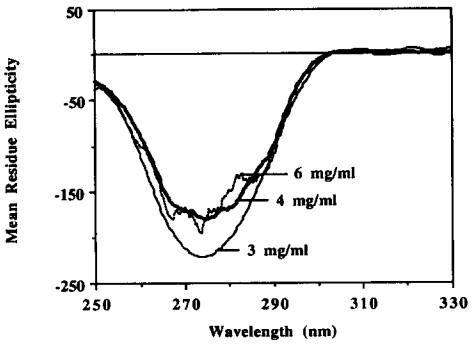

Fig. 7. Concentration dependence of the near UV CD spectrum of LPA in a 4:1 ethanol/water mixture. The solution contained 1% (w/v) of PVP. The pathlengths were either 0.5 or 1 mm and the sample temperature was 25°C.

4. Summary

Circular dichroism spectroscopy has proven to be a powerful tool for examining the solution structure and aggregation behavior of LPA. Although LPA appears to adopt little ordered secondary structure in aqueous solution, upon addition of trifluoroethanol, LPA adopts type II β-turn type structures. These structures are currently being investigated using multi-dimensional NMR spectroscopy.

Current LPA formulations call for the use of nonaqueous solvents, such as ethanol, and high LPA concentrations (Lu et al., 1992). In ethanol/water mixtures, LPA aggregates in a concentration- and temperature-dependent fashion. The aggregates display markedly different NUV CD spectra from monomeric LPA. As NUV CD signals arise from aromatic groups, the particular characteristics of the CD spectrum are dependent on the local environment around each side chain (Manning, 1992). Consequently, the NUV region has been interpreted as indicating the extent of tertiary structure or overall globular folding, although it can also indicate localized denaturation. In the case of LPA, as the peptide molecules associate, the environment around the aromatic

groups changes, and the electronic states of the side chains can interact with additional excited states on adjoining molecules (Manning, 1992). In general, during aggregation, the side chains become more protected from the solvent. Therefore, the NUV CD characteristics begin to vary, depending on the hydrophobicity, solvent accessibility, and degree of order in the immediate vicinity of the chromophore.

Since NUV CD spectra display this sensitivity to change in the local environment, CD spectroscopy can be employed as a sensitive analytical method for detecting the aggregation of peptides. It has been demonstrated that these analyses can be performed even in the presence of certain excipients and under conditions where the aggregates are too small to be detected by light scattering methods.

Acknowledgements

The generous support of this work by Takeda Abbott Pharmaceuticals is gratefully acknowledged. The purchase of the CD spectrophotometer was partially funded by the Colorado Institute for Research in Biotechnology, Abbott Laboratories, and the University of Colorado.

References

Cann, J.R., Cahnnabasavaiah, K. and Stewart, J.M., Circular dichroism study of the solution conformation of luteinizing hormone-releasing hormone. *Biochemistry,* 18 (1979) 5776–5781.

Gray, R.A., Vander Velde, D.G., Burke, C.J., Manning, M.C., Sanyal, G., Middaugh, C.R. and Borchardt, R.T., Delta sleep-induicng peptide: I. Determination of the secondary solution structure and the investigation of the relationship to permeability. *Biochemistry,* 33 (1994) 1323–1331.

Hahn, P.M., Van Vugt, D.A. and Reid, R.L., The stability of synthetic gonadotropin-releasing hormone. *Fertil. Steril.,* 48 (1987) 155–158.

Helm, V.J. and Müller, B.W., Stability of gonadorelin and triptorelin in aqueous solution. *Pharm. Res.,* 7 (1990) 1253–1256.

Holladay, L.A., Circular dichroism of insulin A chain in water: trifluoroethanol mixtures. Use of linear and nonlinear analysis. *Biophys. Chem.,* 7 (1977) 41–49.

Honda, S., Ohashi, S., Morii, H. and Uedaira, H., Solution conformation of human growth hormone-releasing factor fragment (1–29) by CD: characteristic conformational change on phospholipid membrane. *Biopolymers,* 31 (1991) 869–876.

Johnson, D.M., Pritchard, R.A., Taylor, W.F., Conley, D., Zuniga, G. and McGreevy, K.G., Degradation of the LH-RH analog nafarelin acetate in aqueous solution. *Int. J. Pharm.,* 31 (1986) 125–129.

Lehrman, S.R., Tuls, J.L. and Lund, M., Peptide alpha-helicity in aqueous trifluoroethanol: correlations with predicted alpha-helicity and the secondary structure of the corresponding regions of bovine growth hormone. *Biochemistry,* 29 (1990) 5590–5596.

Lu, M.F., Lee, D. and Rao, G.S., Percutaneous absorption enhancement of leuprolide. *Pharm. Res.,* 9 (1992) 1575–1580.

Manning, M.C., Conformation of the alpha form of human calcitonin gene-related peptide (CGRP) in aqueous solution as determined by circular dichroism spectroscopy. *Biochem. Biophys. Res. Commun.,* 160 (1989) 388–392.

Manning, M.C., Protein structure and stability assessment using circular dichroism spectroscopy. In Himmel, M.E. and Georgiou, G. (Eds), *Biocatalyst Design for Stability and Specificity,* ACS Symposium Series, Vol. 516, 1992, pp. 33–52.

Manning, M.C., Patel, K. and Borchardt, R.T., Stability of protein pharmaceuticals. *Pharm. Res.,* 6 (1989) 903–918.

Marche, P., Montenay-Garestier, T., Helene, C. and Fromageot, P., Conformational characteristics of lubirelin. Circular dichroism and fluorescence studies. *Biochemistry,* 15 (1976) 5730–5737.

Matsuura, J. and Manning, M.C., Conformation of human calcitonin gene-related peptide (8–37) in aqueous solution as determined by circular dichroism spectroscopy. *J. Pharm. Biomed. Anal.,* 11 (1993) 89–93.

Motto, M.G., Hamburg, P.F., Graden, D.A., Shaw, C.J. and Cotter, M.L., Characterization of the degradation products of luteinizing hormone releasing hormone. *J. Pharm. Sci.,* 80 (1991) 419–423.

Okada, J., Seo, T., Kasahara, F., Takeda, K. and Kondo, S., New degradation product of des-Gly^{10}-NH$_2$-LH-RH-ethylamide (fertirelin) in aqueous solution. *J. Pharm. Sci.,* 80 (1991) 167–170.

Oyler, A.R., Naldi, R.E., Lloyd, J.R., Graden, D.A., Shaw, C.J. and Cotter, M.L., Characterization of the solution degradation products of histrelin, a gonadotropin releasing hormone (LH/RH) agonist. *J. Pharm. Sci.,* 80 (1991) 271–275.

Perczel, A. and Fasman, G.D., Quantitative analysis of cyclic β-turn models. *Protein Sci.,* 1 (1992) 378–395.

Perczel, A., Hollósi, M., Foxman, B.M. and Fasman, G.D., Conformational analysis of pseudocyclic hexapeptides based on quantitative circular dichroism (CD), NOE, and x-ray data. The pure CD spectra of type I and type II β-turns. *J. Am. Chem. Soc.,* 113 (1991) 9772–9784.

Powell, M.F., Sanders, L.M., Rogerson, A. and Si, V., Parenteral peptide formulations: chemical and physical prop-

erties of Native Luteinizing Hormone-releasing hormone (LHRH) and hydrophobic analogues in aqueous solution. *Pharm. Res.*, 8 (1991) 1258–1263.

Shi, Y.-F., Sherins, R.J., Brightwell, D., Galleli, J.F. and Chatterji, D.C., Long-term stability of aqueous solutions of luteinizing hormone-releasing hormone assayed by an in vitro bioassay and liquid chromatography. *J. Pharm. Sci.*, 78 (1984) 818–821.

Wang, Y.-C.J. and Hanson, M.A., Parenteral formulations of proteins and peptides: stability and stabilizers. *J. Parenteral Sci. Technol.*, 42 (1988) S3–S26.

Winterer, J., Chatterji, D., Comite, F., Decker, M.H., Loriaux, D.L., Galleli, J.F. and Çutler, G.B., Jr, Thermal stability of a long-acting analogue of luteinizing hormone-releasing hormone (D-Trp^6-Pro^9-NEt-LHRH). *Contraception*, 27 (1983) 195–200.

Woody, R.W., Studies of theoretical circular dichroism of polypeptides. In Blout, E.R., Bovey, F.A., Goodman, M. and Lotan, N. (Eds), *Peptides, Polypeptides and Proteins*, Wiley, New York, 1974, pp. 338–350.

Wu, C.-S.C. and Yang, J.T., Sequence-dependent conformation of short polypeptides in a hydrophobic environment. *Mol. Cell. Biochem.*, 40 (1981) 109–122.